GROUPING OF BACILLUS INFLUENZAE BY SPECIFIC AGGLUTINATION

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Attention has recently been directed to the hemophilic bacteria in connection with the much discussed relation of Pfeiffer's bacillus to pandemic influenza. The present grouping of these organisms is unsatisfactory, being based largely on a consideration of the clinical conditions in which they are found. Slight differences in their cultural characters, in their pathogenicity for animals, and meager observations of their immunologic reactions play a secondary rôle in this classification. There has thus been designated: B. influenzae (Pfeiffer,[1] 1892); B. pertussis, the bacillus of acute contagious conjunctivitis, the bacillus of chronic conjunctivitis, a hemophilic bacillus isolated from urine (Davis,[2] 1910), as well as hemophilic organisms from various sources among lower animals. The pseudo-influenza bacillus of Pfeiffer has not been described with sufficient differential characters to warrant its separation from B. influenzae.

Davis,[3] in 1907, studied the immunologic reactions of a number of strains of hemophilic organisms, but was unable to establish by agglutination tests with immune rabbit serum a definite grouping among the strains studied. Park[4] and his co-workers have reported an extensive study of strains of B. influenzae isolated during the recent pandemic of influenza. No grouping of the strains studied was established by agglutination and by the absorption of agglutinins, using the serum of immunized rabbits. Specific agglutination with the homologous serum was obtained, but each strain appeared to be individual in its immunologic reactions. More recently Huntoon and Hannum[5] report work in which they obtained an "almost complete absorption of agglutinins for the immunizing strain and for heterogenous strains," and Roos[6] in collaboration with these workers concludes that "the various strains studied do not differ in kind."

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[1] Deutsch. med. Wchnschr., 1892, 2, p. 465; Ztschr. f. Hyg. u. Infektionskr., 1892, 13, p. 357.
[2] J. Infect. Dis., 1910, 7, p. 599.
[3] Jour. Am. Med. Assn., 1907, 48, p. 1563.
[4] Jour. Am. Med. Assn., 1919, 73,, p. 318.
[5] J. Immunol., 1919, 4, p. 167.
[6] J. Immunol., 1919, 4, p. 189.

We have had the opportunity to make a similar study of ten strains of hemophilic bacteria, all except one of which were typically B. influenzae from their cultural, morphologic, and staining properties. The tenth strain was an organism presenting the cultural and staining characters of B. influenzae, but it exhibited such fantastic pleomorphism that it would scarcely be classed with the B. influenzae group even by those fully appreciating the pleomorphic tendencies of this group. A rabbit was immunized with the organism with the idea of establishing its relationship to the typical strains studied. The results obtained differ from those previously reported in that a definite grouping of the strains studied was obtained.

ISOLATION AND ORIGIN OF STRAINS STUDIED

This study was begun in March, 1919, that is, after the passing of epidemic influenza. The strains were collected from various sources by culture on brown blood-agar plates. Several were from cultures of bronchial secretions made at necropsy at Barnes Hospital. The others were isolated by throat cultures from normal individuals, from influenza patients treated in the different wards of the St. Louis City Hospital, and from patients presenting themselves at the Medical Dispensary of Washington University Medical School.

The colonies of B. influenzae were picked from the mixed cultures after 24 hours' incubation and subcultured on brown blood agar. This medium was used throughout in isolating, in carrying along the strains and in growing the organisms for injection into rabbits and for antigen suspensions. It consisted of beef infusion agar neutral to phenolphthalein to which 5% defibrinated rabbit blood was added at 90 C. immediately before pouring the plates or slants.

IMMUNIZATION OF ANIMALS

Full grown rabbits were immunized by intravenous injection of living organisms from 48-hour growths of the different strains in pure culture. The agar slants or plates used for growing the organisms for the rabbit inoculations were seeded heavily with the different strains. After a 48-hour incubation period, the growth was washed off and emulsified in 5 c c of sterile normal salt solution. One or two cubic centimeters of the suspension was injected immediately into the ear vein of the rabbit. From time to time small amounts of blood were drawn from the ear vein and preliminary homologous agglutination tests were made.

The serums used in the titrations reported were obtained with blood drawn from the rabbit's heart by a syringe. All the animals survived this bleeding, and the yield of serum in every case was sufficient for complete agglutination, cross agglutination and absorption tests. From the protocols it will be noted that after a three or four day interval following the bleeding, the rabbits were reinoculated, using the same size dosage that each rabbit had last received. Within 12 hours following this injection, 4 of the rabbits died. At a previous time 10 c c of blood were drawn from rabbit 2, and immediately an inoculation

was made. This rabbit also died following the inoculation. This experience is cited to emphasize the danger of inoculating large doses following the drawing of considerable blood from immune rabbits.

The blood as drawn was put in sterile centrifuge tubes, placed in the incubator at 37.5 C. for about 10 minutes when the clots were loosened from the sides of the tubes by means of a stiff wire. The tubes were allowed to stand in the icebox over night, then centrifugalized and the serum drawn off.

The following protocols give the details of the strains used and the progress of the animal immunizations.

STRAIN 1. — From necropsy, diagnosis, bronchopneumonia; isolated 4-28-19; antigen planted 5-10-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 1	3/30	1/5 agar slant	
	4/ 6	2/5 agar slant	
	4/12	1/5 agar plate	
	4/22	2/5 agar plate	
	5/ 3	2/5 agar plate	
	5/10	2/5 agar plate	
	5/24	1/5 agar slant	6/5 - 20 c c
	6/ 9	2/5 agar plate	

STRAIN 2.—From the normal throat of a medical student, exposed but no history of influenza; isolated 4-27-19; antigen planted 5-10-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 2	5/13	1/5 agar plate	
	5/16	1/5 agar plate	
	5/20	2/5 agar plate	5/24 bled 10 c c from ear vein
	5/24	2/5 agar plate	

STRAIN 3.—Isolated 4-10-19 from the throat of a girl aged 18 months; diagnosis, influenza; antigen planted 6-6-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 3	4/22	1/5 agar plate	
	4/28	1/5 agar plate	
	5/10	2/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	6/6 - 20 c c from heart
	6/ 9	2/5 agar plate	

STRAIN 4.—From a medical student, ambulatory case of Vincent's angina; isolated 4-25-19; antigen planted 6-7-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 4	4/22	1/5 agar slant	
	4/26	1/5 agar plate	
	5/10	2/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	6/5 - 20 c c from heart
	6/ 9	2/5 agar plate	

STRAIN 5.—Isolated from throat of girl, aged 8; diagnosis, influenza; 4-18-19; planting of antigen, 5-10-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 5	4/26	1/5 agar plate	
	5/ 5	2/5 agar plate	
	5/10	2/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	6/5 - 20 c c from heart
	6/ 9	2/5 agar plate	

STRAIN 6.—From bronchial culture, necropsy, man; no history of influenza; diagnosis, terminal pneumonia following multiple fractures; isolated, 4-25-19; antigen preparation, 6-7-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 6	5/ 5	1/5 agar plate	
	5/10	2/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	6/6 - 20 c c from heart
	6/ 9	2/5 agar plate	

STRAIN 7 (atypical morphologically).—From bronchial culture, necropsy, boy; acute osteomyelitis and staphylococcus septicemia; isolated, 4-19-19; antigen preparation, 6-7-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 7	5/10	1/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	
	5/28	1/5 agar plate	6/6 - 20 c c from heart
	6/ 9	2/5 agar plate	

STRAIN 8.—From throat culture, woman, aged 46; influenza; isolated, 4-10-19; antigen preparation, 6-6-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 8	4/26	1/5 agar plate	
	5/ 5	2/5 agar plate	
	5/10	2/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	6/5 - 20 c c from heart
	6/ 9	2/5 agar plate	

STRAIN 9.—From throat of a man, aged 42; influenza; isolated, 4-12-19; date of antigen preparation, 6-6-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 9	4/26	1/5 agar plate	
	5/ 5	2/5 agar plate	
	5/10	2/5 agar plate	
	5/20	2/5 agar plate	
	5/24	2/5 agar plate	6/5 - 20 c c form heart
	6/ 9	2/5 agar plate	

STRAIN 10.—From throat culture, woman, aged 29; influenza; isolated, 4-10-19; antigen preparation, 5-10-19.

	Injections	Amount 24-Hour Culture	Date of Bleeding
Rabbit 10	4/28	1/5 agar plate	
	5/ 5	2/5 agar plate	
	5/10	2/5 agar plate	
	5/19	2/5 agar plate	
	5/24	2/5 agar plate	6/5 - 20 c c from heart
	6/ 9	2/5 agar plate	

PREPARATION OF ANTIGENS

Twenty-four hour agar-slant cultures were emulsified in 2 or 3 c c of sterile infusion broth, and from this emulsion 15 or 20 petri dish agar surfaces were heavily seeded by means of a sterile cotton swab. After incubating 48 hours at 37.5 C., heavy growths were obtained. This growth was washed off with 0.9% sodium chlorid solution. Small amounts of the salt solution were used in this process, yielding a very heavy bacterial suspension. The operation was much facilitated by the use of a delicate glass scraper made from a capillary pipet by bending it in an "L" shape with the short arm of convenient length, which depended somewhat on the size of the plate surface used. This glass scraper when carefully made presents perfect contact with the agar surface, and can be used in removing the bacterial growth with little danger of removing particles of agar.

The concentrated bacterial suspension thus obtained was collected immediately in sterile centrifuge tubes, and the bacteria precipitated at moderate speed. High speed packs the bacteria too solidly and later renders emulsification more tedious. The supernatant liquid was then drawn off, and the bacteria were suspended in fresh salt solution. In this manner the greater part of the soluble elements from the surface of the agar was removed from the antigen suspension. Precautions to avoid contamination were observed throughout. To the concentrated antigen suspension, 0.5% of liquor formaldehydi was added and the containers thoroughly shaken to effect homogeneous suspension.

The antigen dilution used in the agglutination tests was more or less empirically determined for the first antigen. It was found that a turbidity amounting to little more than a perceptible opalescence in the small agglutination test tube gave very sharp and clear cut agglutination readings. Choosing this dilution as a standard, the other antigens were standardized to it by dilutions and direct comparison, as recommended in the turbidity standardization of antigen in the Dreyer method for agglutination within the typhoid group.

TECHNIC OF AGGLUTINATION TESTS

Agglutination tests were set up with final dilutions of serum of 1:20, 1:40, 1:100, and thence upward by increments of 100 as far as necessary to cover the agglutination zone of the serum tested. The serum dilutions and the antigen were used in 0.5 c c amounts each. The tests were read after 16 hours' incubation at 55 C. Complete agglutination with formation of large clumps is designated + +; complete agglutination with small clumps giving a granular appearance as +, and partial agglutination as ±. Naked eye readings were used, and partial agglutination dilutions disregarded in determining the serum titer.

Table 1 presents the agglutination and cross agglutination values obtained with the serums studied. The numbers given in the table represent the highest dilution which gave the 1 + reading as defined above.

TABLE 1

AGGLUTINATION AND CROSS-AGGLUTINATION TITERS

Immune Serum	Antigens										Normal Rabbit Serum Control	Normal Salt Solution Control
	1	2	3	4	5	6	7*	8	9	10		
1	900	20	40	0	0	40	0	800	700	40	—	—
2	40	1,000	1,900	0	1,400	700	0	0	0	2,000	—	—
3	20	0	20	0	0	0	0	0	0	20	—	—
4	0	0	0	700	0	20	0	0	0	0	—	—
5	40	1,100	1,900	0	1,700	400	0	20	20	1,800	—	—
6	0	100	200	0	200	1,300	0	0	0	400	—	—
7	20	40	40	0	40	600	0	0	0	200	—	—
8	800	0	0	0	0	20	0	600	700	20	—	—
9	400	0	20	0	0	20	0	400	500	20	—	—
10	0	1,300	2,200	0	1,400	100	0	0	0	2,400	±	—

* Antigen not agglutinable.

A definite pro-agglutinoid zone was observed in all the high titer serums. In several instances bacteriolysis was observed in the first dilution. It is suggested that this observation might have some bearing on the explanation of the so-called pro-agglutinoid phenomenon, that is, a sufficient concentration of bacteriolysins occurring along with the agglutinins may impede the action of the latter, or may cause disintegration of the bacterial clumps after they have formed.

DISCUSSION OF AGGLUTINATION TESTS

It will be noted in table 1 that serum 1 agglutinates strains 8 and 9 in about the same dilutions that it agglutinated the homologous strain. Serum 8 agglutinates strains 1 and 9, and serum 9 agglutinates 1 and 8. These three strains act nonspecifically with all other serums, while they appear to cross-agglutinate specifically and suggest their immunologic group identity.

Serum 2 agglutinates strains 3, 5, 6 and 10. In analyzing this group, it is noted first, that serum 3 contains practically no antibodies for its homologous antigen, and consequently must be ruled out as an immune serum.

Serums 5 and 10 contain a high titer of antibodies for strains 2, 3, 5, and 10, while there is a lower titer for strain 6 than appeared in serum 2.

Serum 6 agglutinates 2, 3, 5, and 10, but the antibody titer is low. The cross agglutination tests here place strains 2, 3, 5 and 10 in one group, and leave one in doubt about the placing of strain 6.

Serum 4 agglutinates only its own antigen, and strain 4 is unaffected by any of the other immune serums, suggesting the separate identity of strain 4 among these 10 strains.

Serum 7 shows considerable concentration of antibodies for strains 6 and 10, while the homologous antigen was not agglutinated. Antigen 7 was not affected by any of the immune serums. This result suggests at once that antigen 7 was a nonagglutinable bacterial suspension. This strain, it will be remembered, is the organism classified as "irregular" on account of its pleomorphism, and its great tendency to undergo autolysis. Suspensions of this organism were very difficult to make on account of the difficulty of breakng up the clumps of organisms as they were washed from the agar. That serum 7 contains antibodies is shown by its titer with antigens from other strains, for example 6 and 10.

The agglutination tests suggest:

Group 1.—Strains 1, 8 and 9.

Group 2.—Strains 2, 3, 5, 10, and perhaps 6.

Group 3.—Strain 4 only.

Strain 7 cannot be classified, but appears most nearly related to strains 6 and 10.

ABSORPTION TESTS

The absorption of agglutinins was conducted in the following manner: One cubic centimeter of a 1:10 dilution of the immune serum was mixed with 4 c c of the concentrated antigen in a sterile centrifuge tube. This amount of the antigen was found to be sufficient to absorb the homologous agglutinins after 4 hours' incubation at 37.5 C., the tubes being shaken at half hour intervals. After this period of incubation, the tubes were centrifugalized and the clear supernatant serum dilution (1:50) drawn off. Final dilutions of this were set up 1:100, 200, 400, 800 and 1,600, using all of the antigens which were agglutinated before absorption by the particular serum in dilutions above 1:100. The 16-hour incubation period at 55 C. was again used, and tests read as described under the agglutination tests.

The results of these tests are given in table 2. In the table + indicates an agglutination value, after exposure for absorption approximating serum titer before exposure, while — indicates no agglutination in the lowest dilution used (namely, 1:100). With the high titer serums used failure to show agglutination in 1:100 dilution after exposure for absorption definitely indicates specific absorption of agglutinins.

TABLE 2

AGGLUTINATION WITH TREATED SERUM

Serum	Absorbed with Antigen	Antigen									
		1	2	3	4	5	6	7*	8	9	10
1	10	+	..	..	..	..	..	..	+	+	..
	9	—	..	..	..	..	..	..	—	—	..
2	3	..	—	—	..	—	+	..	..	..	—
3†											
4	6	..	..	..	+	..	..	..	..	..	..
	7	..	..	..	+	..	..	..	..	..	..
5	3	..	—	—	..	—	+	..	..	..	—
	10	..	—	—	..	—	+	..	..	..	—
6	3	..	—	—	..	—	+	..	..	..	—
	4	..	+	+	..	+	+	..	..	..	+
	6	..	—	—	..	—	—	..	..	..	—
	7	..	+	+	..	+	+	..	..	..	+
7	6	..	..	..	..	..	—	..	..	..	—
	4	..	..	..	..	..	+	..	..	..	+
8	1	—	..	..	..	..	..	..	—	—	..
9	1	—	..	..	..	..	..	..	—	—	..
10	1	..	+	+	..	+	+	..	..	..	+
	2	..	—	—	..	—	+	..	..	..	—
	3	..	—	—	..	—	+	..	..	..	—
	5	..	—	—	..	—	+	..	..	..	—
	6	..	+	+	..	(?)	—	..	..	..	+

* Antigen not agglutinable.
† No agglutinins in serum.

The absorption tests definitely established two groups containing in the first instance strains 1, 8 and 9; and in the second, strains 2, 3, 5 and 10.

Strain 4 stood as an individual strain in the agglutination tests. Serum 4 had no agglutinins removed by absorption with strains 6 and 7. Conversely, serum 6 had no agglutinins removed by absorption with strain 4.

Strain 6, which by the agglutination tests showed a relation to the strains of group 2, by the absorption tests is shown to be outside of this group and represents another individual strain. Serum 7 agglu-

tinated strain 6 in a dilution of 1 : 600. When this serum was absorbed with strain 6, this was removed. On account of the inability to prepare a satisfactory agglutinable antigen for strain 7, cross absorption tests could not be done to establsh the relation between strains 6 and 7.

The agglutination tests show some cross group agglutinins between groups 1 and 2, and also between strains 6 and 7, and the two groups. Strain 4 appears more strictly unrelated to the others, its antigen being agglutinated by none of the other serums, and its serum only agglutinating antigen 6 in a 1 : 20 dilution.

SUMMARY

From the results of these experiments it appears that four groups of B. influenzae have been identified, and that 70% of the strains studied fall into two groups:

Group 1.—Strains 1, 8 and 9.
Group 2.—Strains 2, 3, 5 and 10.
Group 3.—Strain 6.
Group 4.—Strain 4.

Strain 7 has not been proved to be distinct from strain 6, and is unclassified.

CONCLUSION

The hemophilic organisms (B. influenzae) can be grouped by immunologic methods, and four groups have been demonstrated.